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Novel insights into microbial community dynamics during the fermentation of Central European ice wine

Mária Bučková[a], Andrea Puškárová[a], Katarína Ženišová[b], Lucia Kraková[a], Ľubica Piknová[b], Tomáš Kuchta[b], Domenico Pangallo[a,c,*]

[a] Institute of Molecular Biology, Slovak Academy of Sciences, Dúbravská cesta 21, 845 51 Bratislava, Slovakia
[b] Department of Microbiology, Molecular Biology and Biotechnology, Food Research Institute, National Agricultural and Food Centre, Priemyselná 4, P. O. Box 25, 824 75 Bratislava 26, Slovakia
[c] Caravella s.r.o., Tupolevova 2, 851 01 Bratislava, Slovakia

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ABSTRACT

Culture-dependent and culture-independent strategies were applied to investigate the microbiota of autumn undamaged and damaged berries, winter berries and ice wine must samples of Grüner Veltliner (Veltlínske zelené) from Small Carpathian wine-producing region. One hundred twenty-six yeasts and 242 bacterial strains isolated from several microbiological media (YPD, PDA, R2A, GYC, MRS and MRS-T) were clustered by ITS-PCR and subsequent Qiaxcel electrophoresis. Representatives of each cluster were identified by sequencing. The extracellular hydrolytic properties and intracellular activities of esterase and β-glucosidase of isolates were assayed. The culture-independent approach permitted the analysis of extracted DNA and RNA coupling DGGE fingerprinting with construction of clone libraries (bacterial and fungal; DGGE-cloning). The combination of the two approaches provided comprehensive data that evidenced the presence of a complex microbiota in each analyzed sample. RNA and DNA analyses facilitated differentiation of living microorganisms from the entire microbiota. Diverse microbial communities colonized the autumn and winter berries. Generally, the combination of results obtained by the methods suggested that the must samples contained mainly *Saccharomyces cerevisiae*, *Metschnikowia* spp., *Hanseniaspora uvarum*, *Lactococcus lactis* and *Leuconostoc* spp. The strains exhibited interesting esterase and β-glucosidase properties, which are important for aroma formation in wine. Fermentation strategies utilising these microorganisms, could be attempted in the future in order to modulate the ice wine characteristics.

1. Introduction

Ice wine is a type of dessert wine that is made from grapes that stayed on the vine longer than a traditional harvest. These grapes are harvested during a hard frost (at around ≤ -7 °C) and pressed while frozen at low temperature. During pressing, much of the water is retained with the grape skins as ice, while a juice highly concentrated in sugars, acids and aroma compounds is extracted (Nurgel et al. 2004). Usually, starter cultures of *Saccharomyces cerevisiae* are used in fermentation (Kontkanen et al. 2004).

The production of ice wine has its origin in Germany between the late of XVIII and the early XIX century. Indeed Germany is the biggest producer of ice wine (Eiswein) in Europe. Besides Germany, there are several Central European countries where the ice wine production was increased in the last decade, offering novel interesting products also from Austria, Czech Republic and Slovakia (Marks 2011).

The commercial success of this kind of sweet wine is growing, but only limited knowledge is available on microbiota occurring during its production and also on the characteristics of isolated strains, in particular of the ice wine produced in Central Europe. The majority of scientific information on ice wine comes from Canada, which is the world leader in the production of this wine. Canadian scientists have investigated many characteristics, interesting yeasts were selected for ice wine fermentation, and their impact on the production of acetic acid, glycerol, and the sensory attributes were also investigated. These studies were carried out with autochthone grapes of these regions such as Riesling, Vidal Blanc (Chamberlain et al. 1997; Crandles et al. 2015; Erasmus et al. 2004) and Cabernet Franc (Synos et al. 2015).

Until now the microbial knowledge of ice-wine derived by cultivation studies and mostly regarding only yeasts. From Canadian ice-wine the following microorganisms were isolated: *Candida* spp., *Hanseniaspora uvarum*, *Aureobasidium pullulans*, *Trichosporon* sp.,

* Corresponding author at: Institute of Molecular Biology, Slovak Academy of Sciences, Dúbravská cesta 21, 845 51 Bratislava, Slovakia.
E-mail address: domenico.pangallo@savba.sk (D. Pangallo).

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Penicillium sp., *Geotrichum* sp., *Fusarium* sp., *Cladosporium* sp., *Paecilomyces* sp., *Trichoderma* sp., *Alternaria* sp., *Pichia kluyveri*, *Rhodotorula* spp., *Cryptococcus* spp., *Issatchenkia terricola*, *Metschnikowia pulcherrima*, *Saccharomycopsis* (*Endomycopsella*) *crataegensis*, *Sporobolomyces* spp., *Sporidiobolus* sp., *Pantoea agglomerans*, *Curtobacterium* spp. and *Pseudomonas corrugata* (Holloway et al. 1990; Subden et al. 2003). A comprehensive investigation using the combination of culture-dependent and culture-independent approaches oriented to both yeasts and bacteria was not applied.

The knowledge of European ice wine is very limited and most of it regards chemical characteristics (Lukić et al. 2016; Setkova et al. 2007). The yeast microbiota of ice wine was studied by the combination of culture-dependent and culture-independent approaches by Alessandria et al. (2013), who investigated the fermentation stages of Mondeuse grape cultivated in north Italy.

No data exist about the microbial communities of ice wine made by some typical Central European grape varieties. The cultivar Veltlínske zelené (Grüner Veltliner) is one of the most cultivated grapes in Central Europe (Robinson et al. 2013). This variety is frequently used for the production of ice wines with different personalities directly connected to the terroirs of the Central European wine region across the borders of Austria, Czech Republic and Slovakia.

In this study, we combined different culture media and culture-independent approaches (DNA and RNA analysis) based on Denaturing Gel Gradient Electrophoresis (DGGE) coupled with clone libraries construction in order to have a view of the microbiota dynamics on grapes and during the different fermentation phases of Veltlínske zelené ice wine.

2. Materials and methods

2.1. Sampling and isolation of microorganisms

The samples were grape berries and must of *Veltlínske zelené variety from Modra (Slovakia)*. Twenty undamaged (G; September 2015), damaged (D; September 2015) and ice berries (W; January 2016) were separately suspended in 100 ml of physiological saline solution (0.9% NaCl) and incubated at laboratory temperature (22 °C) overnight at shaking in order to prepare grape wash.

The ice wine fermentation was carried out spontaneously in a winery located in Modra (Slovakia). The musts were sampled during different stages of the vinification process: middle fermenting must (M1: t = 8 days; sugar = 23° Brix; alcohol = 6%; pH 3.8) and must at the end-fermentation phase, before siphoning (must M2: t = 36 days, sugar = 14.1° Brix; alcohol = 10%; pH 3.3). Ice grapes were harvested at − 6 °C, then they were milled using electrical steel mill with nylon valves. Pressing was carried out by a pneumatic press with a yield of 15–20%. No skin maceration was performed. Vinification took place in 50 l glass vessels without filtration, clarification was carried out using bentonite. The fermentation was carried out without agitation at 16 °C. SO_2 was added to the must at a concentration of 100 mg l^{-1}. As the wine was produced in a traditional way, no starter yeasts, enzymes or additives were used. Fermentation was spontaneous and was not stopped. Decimal dilutions of G, D, W, M1 and M2 in physiological solution were inoculated on different agar media in order to isolate the fungal and bacterial communities. To isolate fungal members Yeast extract Peptone Dextrose (YPD) and Potato Dextrose Agar (PDA; Kraková et al. 2012) were used; while to bacterial strains were used R2A (for the isolation of a broad spectrum of bacteria); Glucose, Yeast extract, Calcium carbonate agar (GYC; for the cultivation of acetic acid bacteria; glucose 50 g l^{-1}, yeast extract 10 g l^{-1}, calcium carbonate 20 g l^{-1}, agar 20 g l^{-1}, 50 mg l^{-1} nisin); de Man–Rogosa–Sharpe (MRS; generic medium for lactic acid bacteria, anaerobic conditions); MRS-Tomato (MRS-T; this medium was used in order to enhance the isolation of lactic acid bacteria, especially *Oenococcus* strains; anaerobic conditions) (Godálová et al. 2016). The media were supplemented either with 80 mg l^{-1} cycloheximide or with 100 mg l^{-1} chloramphenicol to inhibit the growth of fungi and bacteria, respectively. The dehydrated media were purchased from HiMedia (Mumbai, India), the chemicals used for media preparation were provided by Sigma-Aldrich (St. Louis, Missouri, USA). The plates were incubated at 22 °C for 3–10 days. When it was possible, 20–25 colonies were picked up from the plates on the basis of their different morphologies and transferred to new agar plates.

2.2. Clustering and identification of isolated microorganisms

The yeasts were sub-cultured on YPD agar, while the bacterial isolates were maintained on the same medium used for isolation (R2A, GYC, MRS or MRS-T agar).

A loopful of yeast or bacterium freshly grown was suspended in 200 µl of physiological saline solution and centrifuged at 8000*g* for 1 min. The pellet of cells was resuspended in sterile water and centrifuged again at 8000*g* for 1 min. The supernatant was removed, 200 µl of InstaGene Matrix suspension (Bio-Rad, Hercules, California, USA) was added to the pellet and incubated for 40 min at 56 °C. The mixture was vortexed and incubated for 10 min at 100 °C, vortexed again and centrifuged for 3 min at 11000*g*. The supernatant was used as template for PCR analysis.

The isolates were clustered by fungal ITS-PCR [primers: ITS3 (5′-GCA TCG ATG AAG AAC GCA GC-3′); ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′); Kraková et al. 2012] and bacterial ITS-PCR [primers: G17 (5′-GTG AAG TCG TAA CAA GG-3′) and the 1:1 mixture of GplusR (5′-CGT CCT TCA TCG GCT-3′) and GminusR (5′-CGT CCT TCA TCG CCT-3′); Godálová et al. 2016] and the amplicons were separated by Qiaxcel electrophoresis (Qiagen, Hilden, Germany). Representatives of each cluster were identified by sequencing of fungal ITS [primers: ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′); ITS4; Kraková et al. 2012] and by sequencing of bacterial 16S rRNA [primers: 27f (5′-AGA GTT TGA TCM TGG CTC AG-3′); 685r (5′-TCT ACG CAT TTC ACC GCT AC-3′); Godálová et al. 2016]. Before sequencing the PCR products were purified using ExoSAP-IT (Affymetrix, Cleveland, Ohio, USA). Sequencing was performed by a commercial facility (Macrogen, Amsterdam, The Netherlands). The obtained sequences were compared with those present in the GenBank database using a BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The fungal ITS and bacterial 16S rRNA sequences of isolates were deposited in the GenBank database under the accession numbers KY816888-KY816915 and KY816310-KY816389, respectively.

2.3. Culture-independent strategy

2.3.1. DNA and RNA extraction from wine-related samples

DNA and RNA were extracted from 2 ml of the samples G, D, W, M1 and M2. For DNA extraction, the procedure described by Godálová et al. (2016) was followed. RNA was extracted using Spectrum™ Plant Total RNA Kit (Sigma-Aldrich) following the instructions of the producer (protocol B). After elution, the RNA was treated by DNase I (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and part of it directly transcribed to cDNA. The rest of the extracted RNA was stored at − 80 °C. In vitro transcription of RNA was done by Cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, California, USA) according to the protocol of the manufacturer. The obtained cDNA was used as a template for PCR amplification.

2.3.2. PCR amplification of DNA/cDNA

The bacterial 16S rRNA fragment was amplified using primers 27f (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 685r (5′-TCT ACG CAT TTC ACC GCT AC-3′) (Lane 1991). PCR mixture contained 1 × PCR buffer, 2.5 mmol l^{-1} $MgCl_2$, 200 µmol l^{-1} dNTPs, 30 pmol of each primer, 2 U HotStarTaq plus DNA polymerase (Qiagen), and 3 µl of template DNA/cDNA solution in the total reaction volume of 50 µl. The